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(54) Title: USE OF CONOTOXIN PEPTIDES ImI AND MII AS CARDIOVASCULAR AGENTS

(57) Abstract

The present invention is directed to the use of ImI and MII conotoxin peptides, and derivatives thereof, as cardiovascular agents including, but not limited to, heart rate regulating agents, blood pressure regulating agents and anti-arrhythmia agents.

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TITLE OF THE INVENTION

USE OF CONOTOXIN PEPTIDES ImI AND MII AS CARDIOVASCULAR AGENTS

This invention was made with Government support under Grant Nos. GM-48677 and MH-00929 awarded by the National Institutes of Health, Bethesda, Maryland. The United States Government has certain rights in the invention.

BACKGROUND OF THE INVENTION

The present invention is directed to the use of ImI and MII conotoxin peptides, and derivatives thereof, as cardiovascular agents including, but not limited to, heart rate regulating agents, blood pressure regulating agents and anti-arrhythmia agents.

The publications and other materials used herein to illuminate the background of the invention, and in particular, cases to provide additional details respecting the practice, are incorporated by reference, and for convenience are numerically referenced in the following text and respectively grouped in the appended bibliography.

The nicotinic acetylcholine receptors (nAChRs) of skeletal muscle and electroplax are among the best characterized (both in structure and in function) of all ligand-gated ion channels. In contrast, the nAChRs of neurons are less well understood, particularly with regard to their subunit composition *in situ* (Sargent, 1993).

The α-conotoxins belong to a family of structurally related peptides found in the venom of marine snails of the genus *Conus*. These peptides target nAChRs and are promising ligands for investigating different subtypes of nAChRs. Two of these toxins, α-conotoxin MII (from *Conus magus*) and α-conotoxin ImI (from *Conus imperialis*), were recently described, and they discriminate among different combinations of nAChR subunits expressed in *Xenopus* oocytes injected with RNA cloned from rat brain (Johnson et al., 1995, Cartier et al., 1996). The structures of these peptides are set forth below.

In view of these two α-conotoxins' specificities, it was desired to determine whether they could be used to discriminate among nAChRs in an intact physiological system. Thus, the effects of α-conotoxins on nicotinic synaptic transmission in frog sympathetic ganglia were examined. This system is particularly attractive because the ninth and tenth paravertebral sympathetic ganglia of amphibia contain two populations of principal cells, B neurones and C neurones (Nishi et al., 1965;

Dodd & Horn, 1983a; Feldman, 1988). B neurones are larger than C neurones, and B neurones are associated with faster-conducting pre- and post-ganglionic axons. B neurones are innervated by axons in the connective above the 7th ganglion, whereas C neurones are innervated by axons in the rami of 7th and 8th ganglia (see review by Skok, 1973; also Dodd & Horn, 1983a). B and C neurones express both nicotinic and muscarinic ACh receptors. Nicotinic receptors mediate the fast epsp in both classes of neurones; however, the muscarinic receptors in B neurones mediate a slow epsp, and those in C neurones mediate a slow ipsp (Dodd & Horn, 1983b). Finally, fast synaptic transmission in the two classes of neurones in bullfrog is mediated by nAChRs with different channel kinetics (Marshall, 1986; Shen & Horn, 1995; Thigpen, 1995).

The heart does not require innervation in order to beat. However, the heart does have a nervous supply. Cardiac muscle, like smooth muscle and unlike skeletal muscle, has a dual innervation from the autonomic nervous system. Parasympathetic nerves from the tenth cranial nerve (vagus) and sympathetic nerves from a ganglion of the paravertebral trunk both reach the heart. Stimulation of the vagus nerve slows the rate of heart beat, whereas stimulation of the sympathetic nerves to the heart speeds it. The stroke rate at any given time, then, depends upon the ratio of nerve impulse frequencies along these two routes to the heart. In addition, the stroke rate also affects the blood pressure.

The tenth paravertebral sympathetic ganglion has two types of neurones -- B cells and C cells. The nicotinic synapses on B and C cells do have different safety margins, and these safety margins are differentially use-dependent. The nicotinic acetylcholine receptors (nAChRs) of B and C cells have identical sensitivities to d-tubocurarine. The intrinsic safety margins of the nicotinic synapses of B and C neurones are not static but are dynamically influenced by pre- and post-synaptic modulation.

Cardiovascular agents have been developed to treat patients having abnormal heart rate or abnormal blood pressure. It is desired to develop additional cardiovascular agents which are more effective than prior agents and which can discretely affect the heart rate or blood pressure without affecting other muscles.

SUMMARY OF THE INVENTION

The present invention is directed to the use of Imi and MII conotoxin peptides, and derivatives thereof, as cardiovascular agents including, but not limited to, heart rate regulating agents, blood pressure regulating agents and anti-arrhythmia agents.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows a sketch of the recording chamber.

Figure 2 shows that nicotinic antagonists can differentially block synaptic transmission of B and C neurones in the 10th sympathetic ganglion of frog. The figure shows the peak amplitudes of B waves (closed circles) and C waves (open circles) plotted against time. α-Conotoxin ImI (5 μM) almost completely blocked B waves (by 95%), but blocked C waves only slightly (by 21%). α-Conotoxin MI (5 μM) blocked B and C waves by only 26% and 25%, respectively. d-Tubocurarine (dTC, 10 μM) almost completely blocked B waves (by 95%), but blocked C waves only slightly (by 26%). α-Conotoxin MII (5 μM) blocked B waves by 37%, but blocked C waves nearly completely (by > 94%). Dihydro-β-erythroidine (DβE, 5 μM) blocked both B and C waves almost completely (by 95% and > 94%, respectively).

Figure 3 shows traces of responses just before (thin solid curve), during (bold curve) and after (dashed curve) exposure to cholinergic antagonists. The responses were obtained during the experiment illustrated in Figure 2. The horizontal line represents the baseline (zero μV). The stimulus to the preganglionic nerve was presented 5 ms from the start of each trace (see arrow in Figure 3A, note stimulus artifact at this location in all panels). In control responses (thin solid curve in each panel) the first deflection after stimulus artifact is the fast B wave. It is followed by the smaller slow B wave, then the double-humped C wave. Figure 3A shows that α-conotoxin ImI is more effective in blocking B than C waves. Bold response was obtained 18 minutes after addition of 5 μM α-conotoxin ImI, and dashed response after 45 minutes of washing. Figure 3B shows that α-conotoxin MI is not very effective in blocking either B or C waves. Bold response was obtained 55 minutes after addition of 5 μM α-conotoxin MI, and dashed response after 22 minutes of washing. Figure 3C shows that d-tubocurarine is more effective in blocking B than C waves. Bold response was obtained 16 minutes after addition of 10 μM d-tubocurarine, and dashed response after 30 minutes of washing. Figure 3D shows that α-conotoxin MII is much more effected in blocking C than B waves. Bold response was obtained 14 minutes after addition of 5 μM α-conotoxin MII, and dashed response after 40 minutes of washing. Figure 3E shows that dihydro-β-erythroidine blocks B and C waves equally well. Bold response was obtained 8 minutes after addition of 5 μM dihydro-β-erythroidine, and dashed response after 16 minutes of washing. In each panel, lengths of x- and y-axes are 120 ms and 120 μV, respectively.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is directed to the use of ImI and MII conotoxin peptides, and derivatives thereof, as cardiovascular agents including, but not limited to, heart regulating agents, blood pressure regulating agents and anti-arrhythmia agents. ImI and MII have the following formulas:

ImI: Gly-Cys-Cys-Ser-Asp-Pro-Arg-Cys-Ala-Trp-Arg-Cys (SEQ ID NO:1).

The C-terminus is preferably amidated.

MII: Gly-Cys-Cys-Ser-Asn-Pro-Val-Cys-His-Leu-Glu-His-Ser-Asn-Leu-Cys (SEQ ID NO:2).

The C-terminus is preferably amidated.

The proline residues can be substituted with hydroxy-proline without affecting the biological activity of the peptides. In addition, in MII Asn_5 can be substituted with His or Tyr and/or His_{12} can be substituted with Asn or Tyr. Each peptide contains two disulfide bonds between the first and third and the second and fourth cysteine residues.

The identification of the ImI (originally isolated from *Conus imperialis* and further described in McIntosh et al., 1994) and MII (originally isolated from *Conus magus* and further described in Cartier et al., 1996) is described in U.S. Patent No. 5,514,774, incorporated herein by reference. The use of ImI and MII for (a) treating a patient having small-cell lung carcinoma (SCLC); (b) inhibiting SCLC proliferation; (c) detecting the presence of SCLC tumors, and (d) detecting the location of SCLC tumors is described in U.S. Patent No. 5,595,972, incorporated herein by reference.

As described in further detail herein, the effects of two new acetylcholine receptor antagonists, α-conotoxin MII and α-conotoxin ImI, on nicotinic synaptic transmission in the 10th paravertebral sympathetic ganglion of the leopard frog (*Rana pipiens*) were examined. The preganglionic nerve was electrically stimulated (at low frequency, ≤ 1 min^{-1}, to avoid use-dependent changes) while compound action potentials of B and C neurones (B and C waves, respectively) were monitored extracellularly from the postganglionic nerve.

α-conotoxins MII and ImI, at low micromolar concentrations, reversibly blocked both B and C waves. α-conotoxin MII blocked the C wave more effectively than the B wave, whereas α-conotoxin ImI's potency was opposite that of MII's. The observation that nicotinic antagonists can differentially block synaptic transmission of B versus C neurones with opposite selectivities strongly suggests that these neurones possess distinct nicotinic receptors.

In addition to fast and slow B waves described by others, C waves with two temporally distinguishable components were present in the recordings of the present study. Each α-conotoxin

affected fast and slow B waves similarly. Likewise, toxins did not discriminate between the two components of C waves. This suggests that all neurones within each major class (B or C) have the same nicotinic receptors.

The data presented herein clearly shows that Imi and MII differentially block the B and C neurones, and thus are able to differentially block sympathetic impulses to the heart affecting the heart rate and blood pressure. Since these two peptides differentially block these neurones, pharmaceutical compositions are prepared utilizing these activities, depending on the final desired effect. Thus, in certain instances it is desired to administer only one of the peptides, and in other instances it is desired to administer the two peptides together. The use of both peptides enables the blocking of all of the nicotinic sites on these neurones. For example, if it is desired to sustain a prolonged drop in blood pressure, i.e., cardiac output, it will be preferred to use Imi and MII together to block the sympathetic pathway to the heart. In addition, it is possible with the use of the present peptides to discretely block the B and C neurones to exert the desired control over the heart rate and blood pressure without adversely affecting nAChRs of other muscles.

Pharmaceutical compositions containing a compound of the present invention as the active ingredient can be prepared according to conventional pharmaceutical compounding techniques. See, for example, *Remington's Pharmaceutical Sciences*, 18th Ed. (1990, Mack Publishing Co., Easton, PA). Typically, an antagonistic amount of the active ingredient will be admixed with a pharmaceutically acceptable carrier. The carrier may take a wide variety of forms depending on the form of preparation desired for administration, e.g., intravenous, oral or parenteral.

For oral administration, the compounds can be formulated into solid or liquid preparations such as capsules, pills, tablets, lozenges, melts, powders, suspensions or emulsions. In preparing the compositions in oral dosage form, any of the usual pharmaceutical media may be employed, such as, for example, water, glycols, oils, alcohols, flavoring agents, preservatives, coloring agents, suspending agents, and the like in the case of oral liquid preparations (such as, for example, suspensions, elixirs and solutions); or carriers such as starches, sugars, diluents, granulating agents, lubricants, binders, disintegrating agents and the like in the case of oral solid preparations (such as, for example, powders, capsules and tablets). Because of their ease in administration, tablets and capsules represent the most advantageous oral dosage unit form, in which case solid pharmaceutical carriers are obviously employed. If desired, tablets may be sugar-coated or enteric-coated by standard techniques.

For parenteral administration, the compound may be dissolved in a pharmaceutical carrier and administered as either a solution or a suspension. Illustrative of suitable carriers are water, saline, dextrose solutions, fructose solutions, ethanol, or oils of animal, vegetative or synthetic origin. The carrier may also contain other ingredients, for example, preservatives, suspending agents, solubilizing agents, buffers and the like. When the compounds are being administered intrathecally, they may also be dissolved in cerebrospinal fluid.

The active agents can also be administered in a cell based delivery system in which a DNA sequence encoding an active agent is introduced into cells designed for implantation in the body of the patient, especially in the spinal cord region. Suitable delivery systems are described in U.S. Patent No. 5,550,050 and published PCT Application Nos. WO 92/19195, WO 94/25503, WO 95/01203, WO 95/05452, WO 96/02286, WO 96/02646, WO 96/40871, WO 96/40959 and WO 97/12635. The DNA sequence can be isolated from cDNA libraries using degenerate probes based on the sequences of the active agents herein or amplified from these libraries using appropriate degenerate primers. Alternatively, suitable DNA sequences can be prepared synthetically for each of the disclosed active agents on the basis of the disclosed sequences and the known genetic code.

The active agents of the present invention are administered in an amount sufficient to generate the desired cardiovascular effect. The dosage range at which these agents exhibit this effect can vary widely, depending upon the severity of the patient's defect, the patient, the route of administration and the presence of other underlying disease states within the patient. Typically, the active agents exhibit their therapeutic effect at a dosage range from about 0.05 mg/kg to about 250 mg/kg, and preferably from about 0.1 mg/kg to about 100 mg/kg of the active ingredient. A suitable dose can be administered in multiple sub-doses per day. Typically, a dose or sub-dose may contain from about 0.1 mg to about 500 mg of the active ingredient per unit dosage form. A more preferred dosage will contain from about 0.5 mg to about 100 mg of active ingredient per unit dosage form.

The present invention is described by reference to the following Example, which is offered by way of illustration and are not intended to limit the invention in any manner. Standard techniques well known in the art or the techniques specifically described below were utilized.

EXAMPLE 1

Activity of I_{MI} and M_{II} on B and C Neurones

Lumbar paravertebral ganglia 7 through 10 and the adjoining 10th spinal nerve were isolated from adult frogs (*Rana pipiens*) of both sexes. The recording chamber was fabricated from Sylgard, and consisted of a trough about 20 mm long, 3 mm wide and 2 mm deep, which could be divided into four compartments A-D by three partitions 1-3. Three vertical, transverse slits were cut into the wall and floor of the trough with a 10 mm-wide razor blade. The trough was partitioned by inserting into each slot an approximately 8 mm wide x 3 mm high x 0.1 mm thick sheet of Mylar, with a V-shaped notch in the top edge. The bottom edge of each sheet penetrated the floor of the trough so the vertex of the notch rested slightly above the floor. The sympathetic chain was pinned to the bottom of the trough. As shown in Figure 1, ganglia are represented by hatched ovals and the 10th spinal nerve by a broad stippled line. The 7th and 8th ganglia were located in compartment A, and the 9th and 10th ganglia in compartment B. Segments of the 10th nerve (stippled line) containing post-ganglionic axons of neurones in the 10th ganglion traversed partitions 2 and 3. The middle segment of the 10th spinal nerve was located in compartment C, and the caudal stump of the 10th spinal nerve was located in compartment D. Partition 2 effectively reduced the volume of the compartment containing the 10th ganglion and thereby reduced the amount of toxin required; moreover, it helped to minimize stimulus artifacts. Only compartment B (~30 µl volume) was exposed to drugs. The chain of ganglia and the 10th spinal nerve were draped over the notched partitions and pinned to the floor of the trough. A notched Mylar sheet similar to those dividing the trough, but inverted, was slipped into each slot beside the sheet already present. The notches of each of the two Mylar sheets in a given slot were aligned so that the juxtaposed sheets formed a partition or barrier, with diamond-shaped aperture whose size could be varied simply by sliding one sheet past the other. The connective between the 8th and 9th ganglia passed through and occluded the aperture of the first barrier; the aperture of the second barrier was occupied by the 10th spinal nerve caudal to the ramus to the 10th ganglion; and a segment of the 10th spinal nerve near its caudal stump occupied the aperture of the third barrier. Thus, this in all created four ~30 µl compartments, each sufficiently isolated from the next to allow: (1) the fluid in each compartment to be independently maintained, and (2) electrical stimulation or recording across a given Mylar partition.

Platinum wire electrodes were placed on either side of partition 1 and served to convey supramaximal stimuli to preganglionic B and C fibers leading into the 10th (as well as the 9th) ganglion. Postganglionic compound action potentials of B and C neurones in the 10th ganglion (B

and C waves, respectively) were recorded from the 10th spinal nerve with Pt wire electrodes placed on either side of partition 3 and connected to a high-input impedance differential A/C preamplifier (either P-15, Grass Instruments or DAM-50, WP Instruments, with 1 Hz low and 1 kHz high frequency filters). The recording electrode in compartment D led to the positive input of the preamplifier, while that in C led to the negative input. A Pt wire ground electrode was located in compartment B. Stimuli (1 ms rectangular voltage pulses) were provided by a stimulator (S-88, Grass Instruments) through a stimulus isolation unit. Signals were captured with a MacIntosh computer (either an SE/30 or LC III) fitted with an A/D converter (either Lab-LC, National Instruments or MacADIOS adio, GW instruments). Stimuli were triggered, and responses captured (sampling frequency 5 or 10 kHz), displayed, analyzed and stored, with homemade virtual instruments constructed with graphical programming language LabVIEW (National Instruments).

The preparation was bathed in frog Ringer's solution consisting of 111 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 10 mM NaHEPES, pH 7.4. All compartments were static baths except B which contained the 10th (and 9th) ganglion and which could be perfused at a rate of ~0.5 ml/min. To expose the 10th ganglion to drug, the perfusion in compartment B was halted, and the Ringer's within it replaced with that containing the drug. Solutions in all static compartments, including B during drug exposure, were manually refreshed at least every 20 minutes to avoid adverse effects of evaporation.

Synthetic α-conotoxins (McIntosh et al., 1994; Cartier et al., 1996) were used. d-Tubocurarine was from Sigma Chemical Co., and dihydro-β-erythroidine was a gift of Merck& Co. All drugs were dissolved in Ringer's solution. It should be noted that the recording chamber used in these experiments requires as little as 30 µl of test solution, allowing drugs and toxins to be used frugally.

Two features were used to discriminate between responses of B neurones (B waves) and C neurones (C waves); in contrast to C waves, B waves could be elicited with lower stimulus strengths and had shorter latencies (cf., Dodd & Horn, 1983a; Feldman, 1988). Since B and C waves were readily distinguishable in our recordings by their latencies alone, supramaximal stimuli sufficient to elicit both responses were used in the experiments reported here. To minimize possible effects of use-dependent changes in synaptic efficacy, the preganglionic nerve was stimulated at a low frequency of $\leq 1\ min^{-1}$. All experiments were performed at room temperature (10-24 °C).

As previously reported by Feldman (1988), the 10th ganglion of *R. pipiens*, like that of *R. catesbiana* (Dodd & Horn, 1983a), have two types of B waves; a fast one followed immediately by

a slower, smaller one. In addition to these, our recording conditions revealed C waves with two temporally distinguishable components with approximately equal amplitudes and which required similar high stimulus strengths in order to be elicited. The fast and slow B waves as well as the two-component C wave (examples of which ca be seen in Figure 3) were verified to be synaptically mediated since they all were abolished when the 10th ganglion was bathed in Ringer's containing low Ca^{2+} concentration and high Mg^{2+} concentration; furthermore, all were sensitive to nicotinic antagonists as discussed below.

We were specifically interested in the effects that the two new α-conotoxins, ImI and MII, had on nicotinic synaptic transmission through the ganglion. For comparison, we also examined the effects of more conventional nAChR blockers, namely d-tubocurarine and dihydro-β-erythroidine as well as α-conotoxin MI from *C. magus*, which has been known for some time to block neuromuscular nAChRs (Olivera et al., 1985). In all, seventeen 10th ganglia were examined for effects of α-conotoxin MII; subsets of these ganglia were also tested with the other antagonists as indicated below, and the results yielded a consistent picture which is summarized by the experiment illustrated in Figure 2. This figure shows that nicotinic antagonists can differentially block synaptic transmission of B and C neurones in the 10th sympathetic ganglion of frog. The ganglion was exposed consecutively to five different drugs (antagonists; concentration represented by gray line) while action potentials in the postganglionic nerve produced in response to electrical stimulation of the preganglionic nerve (stimulation rate, ≤ 1 min^{-1}) were monitored. The ganglion was washed between exposure to the antagonists. The peak amplitudes of fast B waves and C waves were plotted as a function of time. Both B and C waves slowly declined over the 6.7 hour time span shown. The antagonists were α-conotoxin ImI (5 μM), α-conotoxin MI (5 μM), d-tubocurarine (10 μM), α-conotoxin MII (5 μM) and dihydro-β-erythroidine (5 μM), respectively. Representative response before, during and after exposure to each antagonist are shown in Figure 3.

C waves were more readily blocked by MII than were B waves. For example, in tests involving single trials in five ganglia, 1 μM α-conotoxin MII blocked the fast C wave by 90 ± 10%, while the fast B wave was only blocked by 59 ± 15% (mean ± s.d.). With 5 μM α-conotoxin MII, the fast C wave was blocked by 97 ± 5%, while the fast B wave was blocked by 68 ± 23% (single trials in 6 ganglia). In one ganglion 50 μM α-conotoxin MII was tested, and both B and C waves were blocked by 100%. In all preparations, both fast and slow B waves were flocked equally well by α-conotoxin MII; likewise, both components of C waves were equally sensitive to α-conotoxin MII (see Figure 2D).

In single trials with three ganglia, 5 μM α-conotoxin ImI blocked the fast B wave by 82 ± 5% and the fast C wave by only 14 ± 12 %. Figure 3A shows that although α-conotoxin ImI's effect on the amplitude of the C wave was slight, it noticeably increased the latency of the response. The residual B wave's latency is also delayed. The figure also shows that the sensitivities of both fast and slow B waves to α-conotoxin ImI were very similar, as were the sensitivities of both components of the C wave.

The effects of other nicotinic antagonists on the sympathetic responses were determined in order to compare their effects with those of α-conotoxin MII and ImI. Dihydro-β-erythroidine blocked both B and C waves about equally well; in one ganglion 0.5 μM dihydro-β-erythroidine blocked B waves by 58% and C waves by 46%, and in another ganglion 5 μM blocked both B and C waves by > 90% (see Figure 3E). In two other ganglia 10 μM dihydro-β-erythroidine blocked both B and C waves by 100%. Invariably however, when dihydro-β-erythroidine was washed out, B waves recovered more quickly than did C waves (see Figure 2). Ten μM d-tubocurarine blocked the fast B wave by 98 ± 4% and the C wave by 24 ± 3 % (single trials in 2 ganglia). In this respect, d-tubocurarine's specificity resembles that of α-conotoxin ImI (compare Figures 3A and 3C). α-Conotoxin MI (5 μM) blocked B and C waves only minimally, but noticeably increased the latencies of both waves (see Figure 3B).

In this study, three different *Conus* peptides were used, α-conotoxin MI, MII and ImI. All have structures with a disulfide-bonding framework typical of the α-conotoxin family. α-Conotoxin MI is a well-established blocker of skeletal muscle nAChR's (Olivera et al., 1985). α-Conotoxins MII and ImI have recently been shown to inhibit ACh-gated currents in *Xenopus* oocytes injected with mRNA encoding nAChRs from rat. Thus, α-conotoxin ImI preferably inhibits α7 homomeric complexes expressed in *Xenopus* oocytes (IC_{50} = 0.2 μM), has lower potency for α9 homomeric complexes (IC_{50} = 1.8 μM), and has no effect on receptors composed of other combinations of subunits (Johnson et al., 1995). In contrast, α-conotoxin MII has highest affinity for the α3β2 combination of nAChR subunits (IC_{50} = 0.5 nM) and has at least two orders of magnitude lower potency on other combinations of nAChR subunits (Cartier et al., 1996). However, both α-conotoxins ImI and MII are capable of blocking the nicotinic acetylcholine receptor of frog skeletal muscle (McIntosh et al., 1994; Harris and Yoshikami, unpublished). Our working assumption is that these two α-conotoxins block synaptic transmission in frog ganglia by inhibiting nAChRs, although we have not rigorously ruled out other possibilities. The minimal activity of α-conotoxin MI indicates that the skeletal muscle subtype of nAChR does not play a significant role in synaptic

transmission in sympathetic ganglia, a conclusion consistent with the observation that synaptic transmission in frog sympathetic ganglia, unlike that in skeletal muscle, is not blocked by α-Bungarotoxin (Shen et al., 1994).

The simple method used in the present experiments of monitoring action potentials in the postganglionic nerve provides only an indirect assessment of an antagonist's ability to block nAChRs. The apparent potency of a nicotinic antagonist will depend on the "safety margin" of the excitatory synapse on the neurone; that is, how large the inward synaptic current is relative to that minimally necessary to reach threshold for generation of an action potential. Thus, if the safety margin of the synapses on B neurones were different from that of C neurones, a nicotinic antagonist would differentially block synaptic transmission through these two classes of neurones even if they possessed the same nAChRs. Indeed, Shen and Horn (1995) recently observed that in sympathetic ganglia of bullfrog the nicotinic synapses on B and C cells do have different safety margins; moreover, these safety margins were differentially use-dependent. Furthermore, by measuring synaptic currents under voltage-clamped conditions, Shen and Horn demonstrated that the nAChRs of B and C cells had identical sensitivities to d-tubocurarine. However, our experimental results cannot be explained simply by differences in safety margins because the nicotinic antagonists tested produced not only differential block of B vs. C responses, but they did so with opposite selectivities. That is, whereas α-conotoxin ImI and d-tubocurarine blocked B responses more potently than C responses, α-conotoxin MII did just the reverse. These results strongly suggest the nAChRs of B neurones are pharmacologically distinct from those of C neurones.

The intrinsic safety margins of the nicotinic synapses of B and C neurones would not be expected to be static but instead be dynamically influenced by pre- and post-synaptic modulation such as depression and facilitation (e.g., Shen & Horn, 1995), long-term potentiation (e.g., Minota et al., 1991), and the activities of homosynaptically-activated muscarinic receptors as well as hererosynaptically-activated peptidergic receptors (e.g., Jan and Jan, 1982; Kuffler & Sejnowski, 1983; Horn, 1992). Factors such as these may be responsible tor the variability we have observed in the sensitivities of responses to a given antagonist in different ganglia. Such variations from one ganglion to the next were also witnessed by Shen et al. (1994, see their Figure 2B), who examined the effects of neuronal bungarotoxin on nicotinic transmission in bullfrog sympathetic ganglia by an assay similar to ours. This discouraged us from attempting to obtain quantitative dose-response relationships for the α-conotoxins with the present extracellular assay. A more quantitative, *albeit* considerably more involved, assay would be to voltage-clamp neurones and monitor spontaneous

miniature epsc's (e.g., Thigpen, 1995) or the responses to exogenously applied ACh (e.g., Akaike et al., 1989). This would provide a direct measure of an antagonist's effect on AChR's. Use of such methods to characterize the toxins' effects remains for future experiments.

Our observations with d-tubocurarine are consistent with those of Nishi et l. (1965), who noted that in toad ganglia the orthodromic response of C neurones was more resistant to d-tubocurarine than was that of B neurones. In contrast, Shen and Horn (1995) observed the opposite in bullfrog ganglia stimulated at low frequency; i.e., the C (compared to B) wave is more sensitive to d-tubocurarine. The effects of the α-conotoxins on B and C neurones in ganglia of these other anuran species remain to be compared.

The various nicotinic antagonists did not discriminate between fast and slow B waves, indicating that the same subtype of nAChR may be shared by all B neurones. Likewise, the two components of the C wave were not differentially blocked by the antagonists, suggesting that the neurones responsible for the two-component C wave all have the same subtype of nAChR. An increase in the latencies of partially blocked B and C waves was observed regardless of the antagonist producing the block (see Figure 3), and it is likely to be a reflection of an increased time required for the attenuated nicotinic epsp to reach threshold to generate the action potential.

With regard to the two-component C wave, it is interesting to note that Nishi et al. (1965) observed that sympathetic axons in sciatic nerve of toad (*Bufo vulgaris japonica*) had three distinct conduction velocities and classified them as B, C_1 and C_2 in view of their belief that C_1 and C_2 axons belong to C cells (but see Dodd & Horn, 1983). Perhaps C_1 and C_2 are the toad counterpart of the two-component C waves we observe.

Marshall (1986) reported that B and C neurones in bullfrog sympathetic ganglia have kinetically distinct nAChRs, with C neurones having nAChRs with a two-fold longer mean open time that those of B neurones. Furthermore, B neurones, which had been surgically denervated and reinnervated by preganglionic C fibers instead, were found to have nAChRs with channel kinetics normally associated with those of C neurones (Marshall, 1985). In view of our results, it would be interesting to see whether the pharmacology of the nAChRs of the neurones are also altered when their synaptic inputs are switched.

The prey of C. *magus*, whose venom contains α-conotoxin MII, is fish. Although α-conotoxin MII blocks skeletal nAChRs in frog (Harris & Yoshikami, unpublished), injection of α-conotoxin MII intramuscularly into goldfish does not cause paralysis (Cartier et al., 1996). This observation, in conjunction with the results reported here, tempts one to speculate that perhaps α-

conotoxin MII's role in *C. magus* venom might be to specifically inhibit the sympathetically mediated flight-or-fight response of fish prey, in contrast to the other toxins in C. *magus* venom, such as α-conotoxin MI and ω-conotoxin MVIIA, that paralyze fish by blocking neuromuscular transmission (Olivera et al., 1985). In this regard, it may be notable that, as evident in Figures 2 and 3, α-conotoxin MI appears to be relatively ineffective in blocking ganglionic nAChRs.

It will be appreciated that the methods and compositions of the instant invention can be incorporated in the form of a variety of embodiments, only a few of which are disclosed herein. It will be apparent to the artisan that other embodiments exist and do not depart from the spirit of the invention. Thus, the described embodiments are illustrative and should not be construed as restrictive.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: University of Utah Research Foundation

(ii) TITLE OF INVENTION: Use of Conotoxin Peptides ImI and MII as Cardiovascular Agents

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
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(F) ZIP: 20004

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
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(B) FILING DATE:
(C) CLASSIFICATION:

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(A) APPLICATION NUMBER: US 60/031,141
(B) FILING DATE: 18-NOV-1996

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(C) REFERENCE/DOCKET NUMBER: 2314-115PCT

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 202-783-6040
(B) TELEFAX: 202-783-6031

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Gly Cys Cys Ser Asp Pro Arg Cys Ala Trp Arg Cys
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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1 5 10 15

WHAT IS CLAIMED IS:

1. A method for treating a patient having an altered heart rate by administration of a therapeutically effect amount of an active agent selected from the group consisting of Imi, Mii, derivatives thereof, and mixtures thereof.

2. The method of claim 1, wherein the active agent is administered in an amount from about 0.05 mg/kg to about 250 mg/kg.

3. The method of claim 1, wherein the active agent is administered in an amount from about 0.1 mg/kg to about 100 mg/kg.

4. A method for treating a patient having an altered blood pressure by administration of a therapeutically effective amount of an active agent selected from the group consisting of Imi, Mii, derivatives thereof, and mixtures thereof.

5. The method of claim 4, wherein the active agent is administered in an amount from about 0.05 mg/kg to about 250 mg/kg.

6. The method of claim 4, wherein the active agent is administered in an amount from about 0.1 mg/kg to about 100 mg/kg.

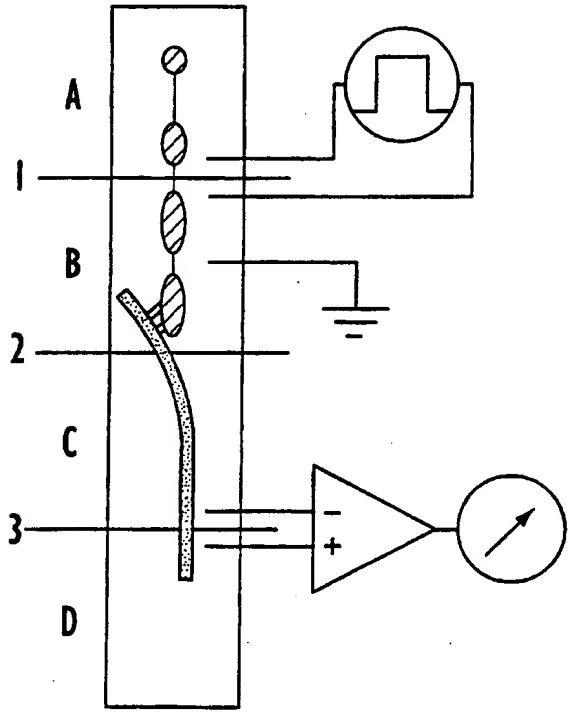

FIG. 1

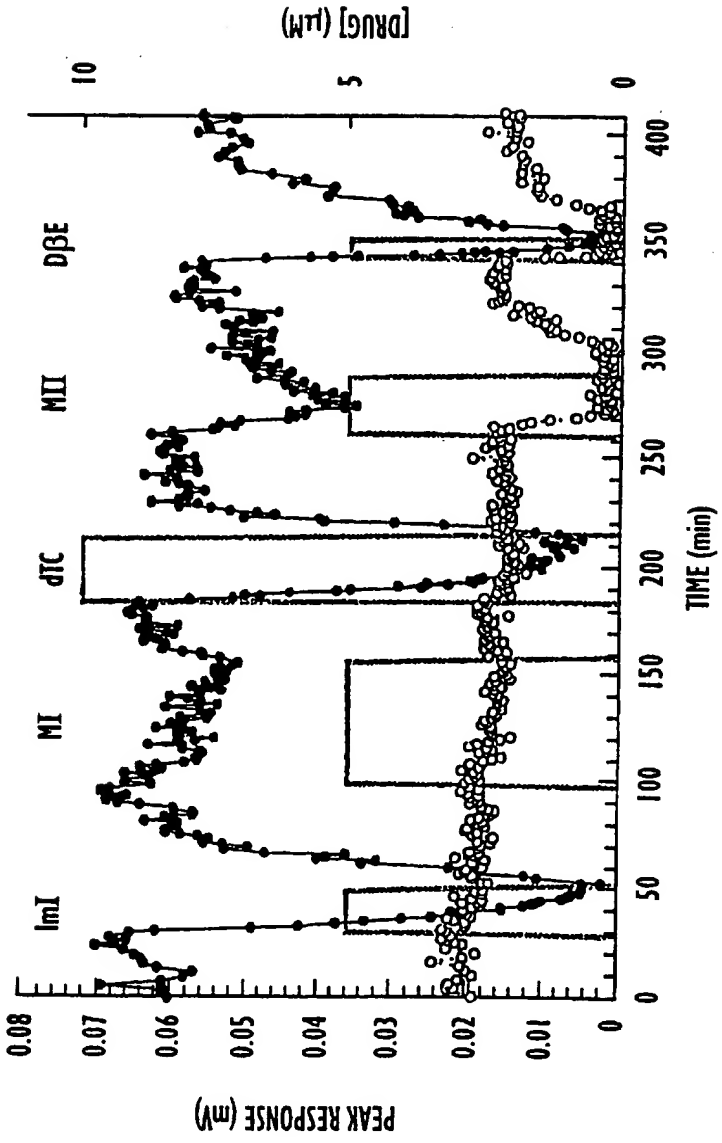
[DRUG] (μM)
10
5
0
ImI
MI
dTC
MII
DβE
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
50
100
150
200
250
300
350
400
TIME (min)
PEAK RESPONSE (mV)

FIG. 2

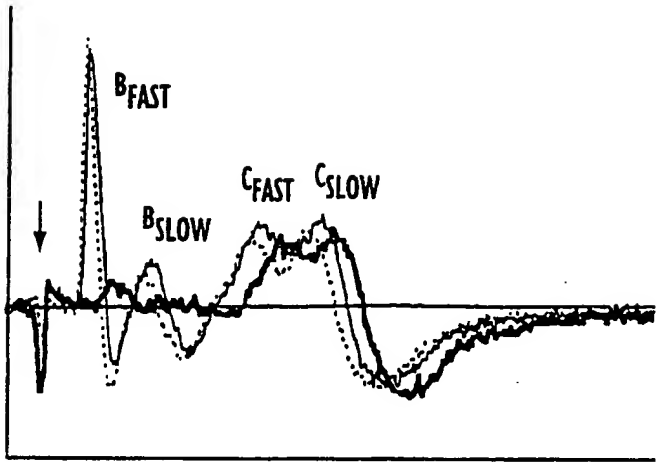

FIG. 3A

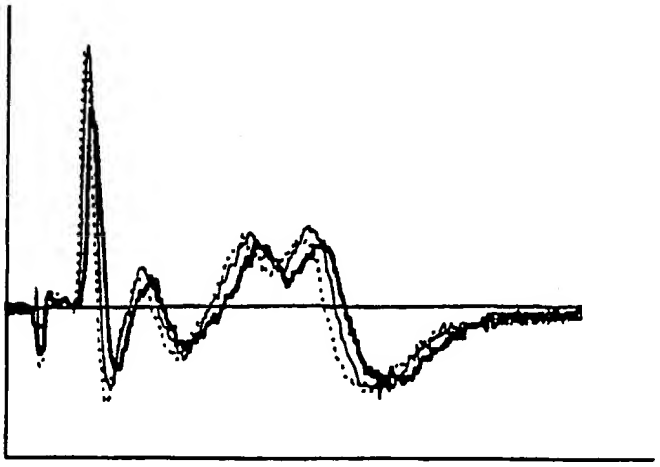

FIG. 3B

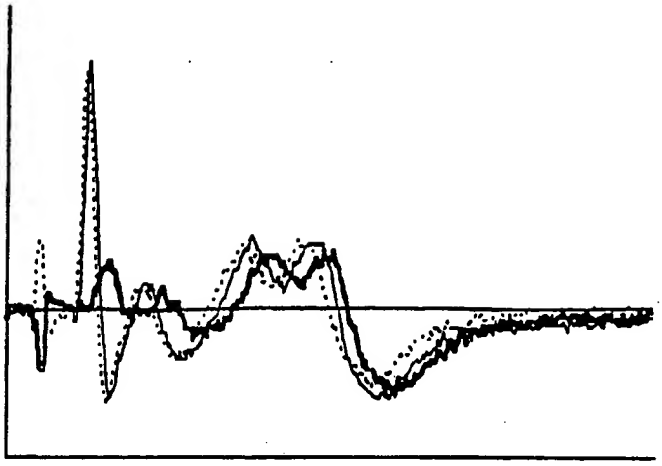

FIG. 3C

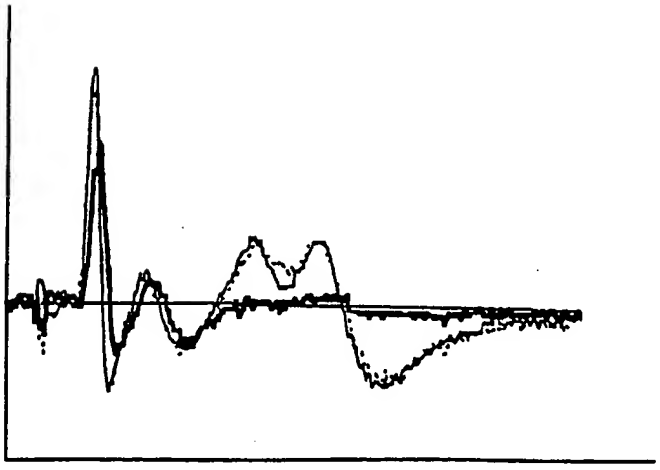

FIG. 3D

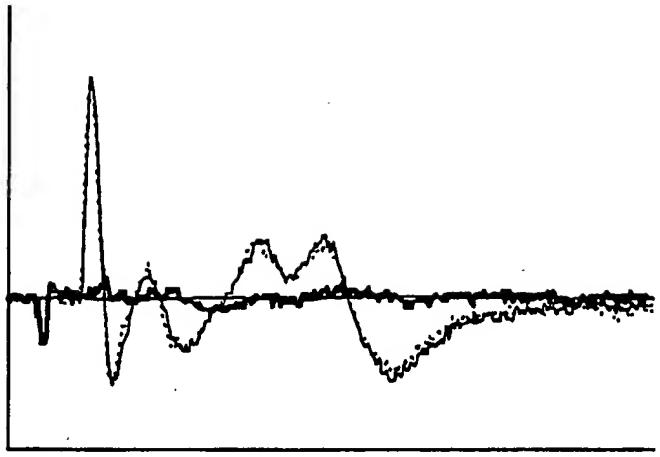

FIG. 3E

INTERNATI NAL SEARCH REPORT

International application No.
PCT/US97/20669

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 38/10
US CL :530/326, 327

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/326, 327

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS Online

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,189,020 A (MILJANICH et al) 23 February 1993, see entire document.	1-6

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
03 FEBRUARY 1998	25 FEB 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer DAVID LUKTON Telephone No. (703) 308-0000

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